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Induction of a Pregnancy-Like Mammary Gland Differentiation by Docosapentaenoic Omega-3 Fatty Acid

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14. ABSTRACT

The protective effect of early pregnancy against breast cancer can be attributed to the transition from undifferentiated cells in the nulliparous to the differentiated mature cells during pregnancy. Considerable evidences suggest strongly that the n-3 polyunsaturated fatty acid (PUFA) content of adipose breast tissue is inversely associated with an increased risk of breast cancer. Here we report that there was a decrease in n-6/n-3 PUFA ratio and a significant increase in concentration of n-3 PUFA DPA and EPA in the pregnant gland. The functional role of n-3 PUFAs on differentiation was supported by the studies in fat-1 transgenic mouse, which converts endogenous n-6 to n-3 PUFAs. Thus, alternation of n-6/n-3 fatty acid compositional ratio in favor of n-3 PUFA and particularly DPA and EPA is one of the underlying mechanisms of pregnancy-induced mammary differentiation. DPA induced mammary gland functional differentiation is dependent on Jak2-Stat5 signaling. Treatment of experimental animals with DPA reduces carcinogen-induced mammary tumorigenesis.

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INTRODUCTION

A1. Controversy over the association between omega-3 fatty acids (n-3 PUFA) and lower risk of breast cancer. Although experimental animal studies indicate that including n-3 PUFAs in the diet has cancer preventive benefits, findings from case-control and cohort studies have **inconsistently** reported an association between questionnaire-based assessment of n-3 PUFA intake and breast cancer risk (1-2). In a recent meta-analysis, Saadatian-Elahi (2) analyzed 11 case-control and 3 cohort studies published in 1966-2002 and reported an inconsistent finding from case-control studies but suggested that data from cohort studies support a significant inverse association between n-3 PUFA and breast cancer risk. The most recent systematic review of 20 cohorts with different demographic characteristics by MacLean also suggests that there is **no significant association** between n-3 PUFA and cancer incidence (1). However, results from studies of n-3 PUFA concentrations in **adipose tissue** (3-7) and **erythrocytes** (8-9) were more consistent. In **mammary** tissue, a variety of evidences suggest strongly that the content of n-3 PUFA in adipose breast tissue is **inversely** associated with increased risk of breast cancer incidence and progression. The most comprehensive study came from the European Community Multicenter, in which the fatty acid contents of adipose tissue in postmenopausal breast cancer cases and controls were analyzed in five European countries (3). The study showed a significantly lower ratio of n-3 to n-6 PUFA in breast cancers vs. controls. Recent study by Bougnoux et al (7) also found that a composite measure of a low ratio of n-3 to n-6 fatty acid in adipose tissue was associated with increased risk of breast cancer. Two most recent large scale case-control studies in China and Japan clearly demonstrated that level of n-3 PUFA, **measured in erythrocytes**, is significantly associated with l**ower risk** of breast cancer (8-9).

We believe that studies from questionnaire-based assessment of fatty acid intake should be interpreted with caution. First, studies on n-3 PUFA consumption varied a great deal across study cohorts. Second, interpretation of the data is limited by significant differences in the methods used to ascertain exposure to n-3 PUFA. Third, of particular note is the fact that n-3 PUFA consumption generally consists of varying the ratio of n-3 to n-6 PUFA without consideration of n-6 fatty acid consumption. Very importantly, when calculating n-3 PUFA consumption, the background n-6 PUFA consumption has to be considered. It is becoming more accepted that most of the beneficial effects including cancer prevention is mediated by alternation of the n-6/n-3 compositional ratio but not the exact amount of n-3 PUFA. It is important to emphasize that all studies in these systematic analysis were prospective in design and no randomized clinical trial data exist.

A2. Two unique transgenic models to study the effect of n-6/n-3 PUFA compositional ratio on mammary gland differention (see Appendix 1-2).

A2-1. N-6/n-3 fatty acid ratio in Fat-1 transgenic mice. Mammals are unable to produce n-3 PUFAs from the more abundant n-6 type, so have to rely on their diet for these nutrients. A unique **fat-1** gene was identified in the roundworm *Caenorhabditis elegans*, which can catalyze the n-6 to n-3 conversion. A **fat-1 transgenic** mouse model capable of converting n-6 fatty acids to n-3 fatty acids was recently established (10). When fed with a diet high in n-6 and low in n-3 fatty acids, the transgenic animals are characterized by an abundance of n-3 fatty acid and a balanced n-6/n-3 fatty acid ratio of **2-1:1** in their tissues and organs, whereas wild type mice have a ratio of **> 30** (10). This model allows one to produce two different fatty acid profiles (high vs. low n-6/n-3 ratios) in the animals by using just a single diet, which avoids the potential problems associated with dietary supplement of fish oil including various amount of different n-3 PUFAs and contaminants. Using this transgenic model, we demonstrated that the ratio n-6/n-3 PUFA in mammary gland was **dropped 12-fold** from 25 in wild type mice to 2 in transgenic mice (**Appendix 1**).

A2-2. MRG transgenic mice: capable of preferential accumulation of n-3 PUFAs into mammary gland (**Appendix 1 and 2**). Mammary derived growth inhibitor Related Gene (**MRG**). We have previously identified, cloned, and characterized a differentiation factor and a fatty acid binding protein MRG in human mammary gland (11-13). Interestingly, MRG revealed no homology to any other known growth

inhibitors; rather, they revealed extensive sequence homology to **fatty acid binding protein** (**FABP**) (14). The sequence of **MRG** was found to be **identical** to the later deposited sequences of human brain type **(B-) FABP** in GenBank (accession #AJ002962) (14). Cellular FABP comprise a well-established family of cytoplasmic hydrophobic ligand binding proteins and are involved in binding and intracellular transport of PUFAs (15). Brain has the highest content of n-3 PUFA or lowest n-6/n-3 ratio among all the tissues (16-17). Preferential accumulation of n-3 PUFA in brain is associated with abundant expression of MRG/B-FABP (16-17). Indeed, among many fatty acids, n-3 PUFAs and particularly DHA has highest ligand binding affinity for MRG (K_d 10 nM) (18). Using previously established MRG transgenic mouse (13), we demonstrated that expression of MRG in mammary gland results in significant alternation of n-6/n-3 PUFA compositional **ratio** in favor of n-3 PUFAs (**Appendix 2**).

BODY

A notable finding relevant to this study is that there is a decrease in n-6/n-3 ratio in the pregnant gland compared with the virgin gland. Of specific interest is the observation of a robust increase of a specific n-3 DPA in differentiated pregnant gland from non-detectable in virgin gland to an abundant accumulation in the pregnant gland. Alternation of n-6/n-3 ratio in favor of n-3 PUFA in the mammary gland of fat-1 transgenic mouse induces differentiation and also significantly increases the DPA accumulation. Expression of MRG, a previously identified mammary differentiation factor and also a brain type fatty acid binding protein (B-FABP), in the transgenic mouse results in mammary differentiation and a robust DPA accumulation. We **hypotheses** that n-3 DPA is one of the mediators in the differentiation effect of pregnancy on breast epithelial cells; and thus application of n-3 DPA to mammary gland can lower breast cancer risk by making the mammary epithelial cells behave like the glands during pregnancy

SA1. Determine molecular mechanism for induction of mammary differentiation by n-3 PUFA DPA (Finished). One of the hallmarks for functional mammary differentiation is the expression of milk protein β-casein, which is mediated by phosphorylation of Stat5 (36-37). Consistent with induction of mammary differentiation by n-3 PUFAs, DPA activates Stat5 and induces β-casein. We will determine if induction of mammary differentiation by individual n-3 PUFAs, primarily DPA that preferentially accumulated in the differentiated gland during pregnancy, is mediated by activation of Jak2-Stat5 signaling pathway.

SA1-1. Stimulation of β-casein expression and induction of Stat5 activation. In mammary gland development, the alveolar buds represent a developmental pathway that eventually leads to secretary alveoli during differentiation. To determine if the mammary epithelial cells were functionally as well as morphologically differentiated, the expression of the early differentiation marker milk protein β-casein was analyzed by real time RT-PCR. Fig. 1A shows representatives of β-casein expression in two virgin control mice and two age-matched virgin fat-1 mice. Whereas minimal levels of β-casein were detectable in non-differentiated virgin mice, increasing n-3 PUFAs composition in the fat-1 mammary gland significantly enhanced β-casein expression, resulting in an average 6.5-fold increase over control mice. These results indicate that the mammary glands of the fat-1 mice have the morphological formation of alveolar-like structure and functional expression of the early differentiation marker β-casein. The histological as well as molecular change observed in the gland from the transgenic mice resembles the differentiated phenotype in the gland from the early pregnant mice.

The transcriptional activation of β-casein gene expression in mammary gland is mediated at least in part by Jak2 and Stat5 pathway. Phosphorylation on tyrosine is essential for Stat 5 binding and

its transcriptional activity. We examined tyrosine phosphorylation of Stat5 in mammary glands of virgin control mice and virgin transgenic mice (Fig. 1B). Whereas undetectable or very limited phosphorylated Stat5 protein was observed in the gland from the non-differentiated virgin control mice, Stat5 phosphorylation was significantly increased in the mammary gland from virgin fat-1 mouse. These data demonstrated that alternation of n-6/n-3 compositional ratio in favor of n-3 fatty acid results in a phosphorylation of Stat5, indicating a potential role of n-3 fatty acid in activating of Stat5 in the mammary gland and induction of mammary gland differentiation.

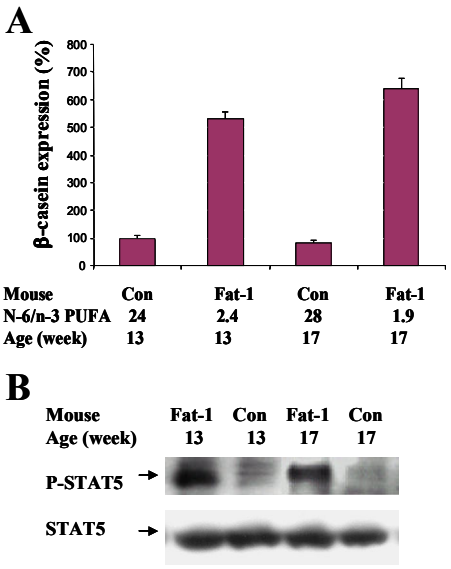
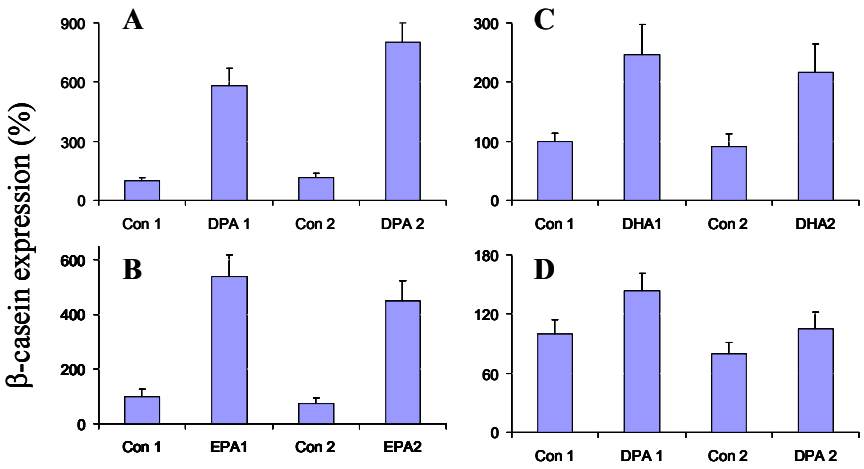


Fig. 1. Molecular analysis of mammary gland differentiation in fat-1 mice. **A**. Quantitative RT-PCR analysis of β-casein expression. Inguinal mammary glands were isolated from age-matched virgin control and fat-1 mice. RNA was isolated and subjected to real time PCR analysis. Relative expressions of mouse β-casein gene in the mammary glands from fat-1 mice were calculated in comparison to that from control mouse. The β-casein gene expression in the 13-week old control mouse was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples. Statistical comparisons for both fat-1 mice relative to control mice indicate $p < 0.001$ for the relative β-casein expression. **B**. Induction of Stat5 phosphorylation in the mammary glands of fat-1 transgenic mice. Thirteen-and 17-week old virgin control mice and age-matched transgenic mice were sacrificed, inguinal mammary glands were removed. Total protein was isolated, normalized, and 300 μg of total protein was subjected to immunoprecipitation with anti-Stat5 antibody followed by Western analysis. The expression of phosphorylated Stat5 was determined by using a specific anti-phosphorylated Star5 antibody and normalized for total Stat5 expression.

SA1-2. Is n-3 PUFA-induced pregnancy like mammary differentiation dependent on Stat5 activation? We used an *ex vivo* model involving mouse whole-organ culture of mammary gland to study whether n-3 PUFAs DPA, EPA, and DHA can regulate milk protein β-casein. Inguinal mammary glands from virgin mice were cultured for 6 days with or without 30 μM DPA, or EPA, or DHA. Consistent with the observed differentiated phenotype in the transgenic gland, a differentiation with stimulation of β-casein was observed in the glands treated with DPA. Expression of β-casein mRNA was significantly increased in DPA treated glands with an average 6.4-fold increase over the control non-treated glands (Fig. 2A). A similar significant stimulation of β-casein expression was also observed in EPA-treated glands, resulting in a 5.7-fold increase over controls (Fig. 2B). Treatments of glands with DHA resulted in a slight increase (2.4-fold) in β-casein expression over controls (Fig. 2C).

To functionally validate the role of Start5 on n-3 PUFA-induced mammary differentiation, we examined the effect of DPA on induction of β-casein expression on *ex vivo* model using mammary glands from Stat5a-deficient $Stat5a^{tm1Mam}$ mice (29). In $Stat5a^{tm1Mam}$ mice, mammary ductal development through pregnancy is normal, but lobuloalveolar development is severely reduced and there is no milk secretion even after prolonged suckling. Whereas DPA induced a significant stimulation of β-casein expression in the glands from wild type mice (Fig. 2A), there were only a slight increase but not significant in DPA-treated Stat5 knock out glands (Fig. 2D). These data indicate that the preferential accumulation of n-3 PUFAs, such as DPA and EPA, in the differentiated mammary gland during pregnancy may act as a factor inducing functional mammary gland differentiation mediated by activation of Jak2 and Stat5.

Fig. 2. Stimulation of β-casein expression by n-3 PUFAs. Two pairs of inguinal mammary glands from two 14-week wild type virgin non-transgenic control mice (**A-C**) and Stat5a knockout mice ***Stat5a^{tm1Mam}*** (**D**) were cultured for 6 days with or without 30 μM DPA (C and F), or EPA (D), or DHA (E) in the organ culture medium. Fresh media containing n-3 PUFAs were added every two days. At the end of 6-day treatment, the gland was subjected to RNA extraction for RT-PCR analysis of β-casein expression. Relative expressions of mouse β-casein gene in the mammary glands treated with n-3 PUFAs were calculated in comparison with that from Con 1 mouse, which was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples.



SA1-3. Determine if activation of Stat5 by n-3 DPA requires Jak2

We used the mammary glands from Jak2 conditional knockout mice to functionally determine if DPA-induced mammary gland differentiation is mediated by activation of Jak2. Whereas DPA induced a significant stimulation of β-casein expression in the glands from wild type mice (Fig. 3A), treatment of the glands from Jak2 conditional knockout mice failed to induce β-casein expression. (Fig. 3B). These data indicate n-3 PUFAs-induced mammary gland functional differentiation is dependent on Jak2 function.

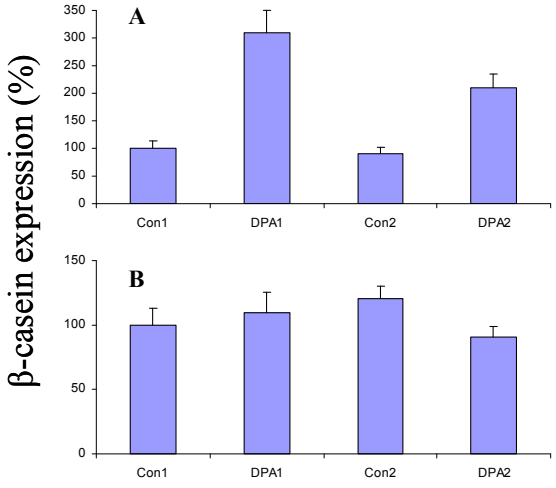


Fig. 3. Stimulation of β-casein expression by n-3 DPA. Two pairs of inguinal mammary glands from two 14-week wild type virgin non-transgenic control mice (**A)** and Jak2 conditional knockout mice (UneMed) (**B**) were cultured for 6 days with or without 30 μM DPA in the organ culture medium. Fresh media containing DPA were added every two days. At the end of 6-day treatment, the gland was subjected to RNA extraction for RT-PCR analysis of β-casein expression. Relative expressions of mouse β-casein gene in the mammary glands treated with DPA were calculated in comparison with that from Con 1 mouse, which was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples.

SA1-4. Determine if activation of Stat5 by n-3 DPA requires a reduction of Jak2-Stat5 inhibitor cavolin-1.

We used mammary glands from Cav1 knockout mice *Cav1^{tm1Mls}* (**Jackson Lab**) and determined β-casein in control (non-treated) and DPA-treated mammary gland in organ culture. Treatment of the glands from Cav1 knockout mice did not affect β-casein expression. (Fig. 4). Since Cav1 was found to associate specifically with Jak2 and **down-regulate** Jak2-Stat5a signaling, and we

demonstrated that treatments of glands from *Cav1*^{tm1Mls} mice with n-3 PUFA DPA do not change the status of β-casein expression, these data suggest that down-regulation of Cav1 plays a critical role for DPA-mediated Stat5 activation and gland differentiation.

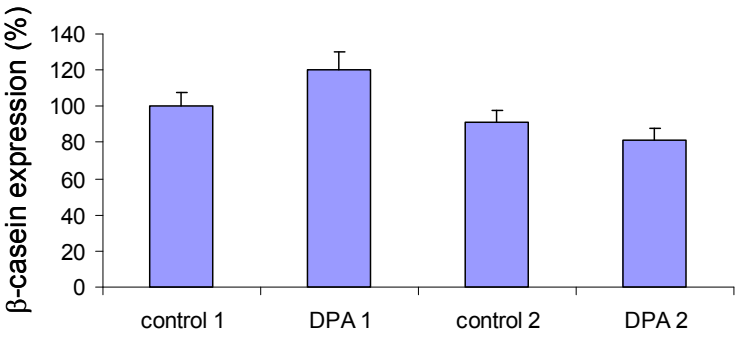


Fig. 4. Effect of DPA on β-casein expression. Two pairs of inguinal mammary glands from two 14-week *Cav1*^{tm1Mls} mice were cultured for 6 days with or without 30 μM DPA in the organ culture medium. Fresh media containing DPA were added every two days. At the end of 6-day treatment, the gland was subjected to RNA extraction for RT-PCR analysis of β-casein expression. Relative expressions of mouse β-casein gene in the mammary glands treated with DPA were calculated in comparison with that from Con 1 mouse, which was taken as 100% and regarded as control. All the other values were expressed as a percentage of the control. The mouse beta actin gene was used as endogenous control. The numbers represent the means ± SD of duplicate samples.

SA2. Does DPA prevent development of preneoplastic lesions and mammary tumorigenesis? (Finished). For cancer prevention studies using n-3 PUFA, most of the experiments use crude marine oil with a various different classes of n-3 PUFAs. Although EPA, DPA, linolenic acid, and DPA are considered as a group of n-3 PUFA, there is an urgent need for further investigation of the precise activities of each individual n-3 PUFAs on mammary differentiation and breast cancer prevention. Unlike DHA and EPA, which are widely available and abundant in fish oil and extensively studied, there are much less functional studies for DPA. We will test the hypothesis that n-3 DPA, an under-explored n-3 PUFA preferentially accumulated in the pregnant gland, mimics the pregnancy effect on differentiation and prevention of mammary tumor.

SA2-1. Mouse mammary organ culture (MMOC) mammary carcinogenic model. MMOC model is useful for studying mammary tumorigenesis because glands in the organ culture exposed to carcinogen DMBA develop hyperplastic lesions. We used this model to study if DPA can prevent DMBA-induced mammary hyperplasia. As demonstrated in Fig. 5, glands at 25 days following DMBA treatment show a hyperplasia-like structure (Fig. 5B). In contrast to the ducts in normal gland (Fig. 5A), which are largely lumina lined with one or two layers of epithelial cells, preneoplastic lesions in DMBA-treated gland are typical hyperplasia, thickened and lined with multiple layers of hyperplastic cells (Fig. 5B). In addition, epithelium of DMBA-treated glands exhibits a disorganized structure with respect to the ordinate arrangement of the wild-type epithelium. Pretreatment of the glands with DPA did not affect the formation of DMBA-induced hyperplasia (Fig. 5C).

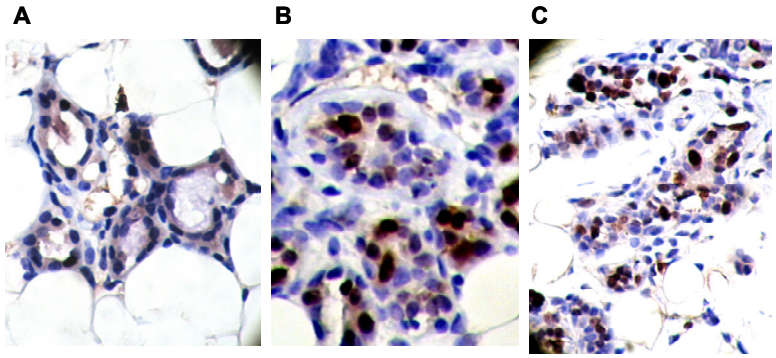


Fig. 5. Mammary hyperplasia in organ culture. Briefly, glands from 10-week of virgin mouse were first treated with or without 50 μM of DPA for 6 days. DMBA (2 μg/ml) was added for 24 hours on day 7. After the 24-h DMBA treatment, the glands are either continually treated with DPA or just cultured in the basal MMOC medium without any treatment. After a

total of 25 days, glands are fixed, stained with H&E, and processed for histopathological evaluation of preneoplastic lesions. **A**, control gland. **B**, DMBA-treated. **C**, gland treated with DPA and DMBA.

SA2-2. DMBA-induced mammary tumors. It has been previously demonstrated that n-3 PUFA EPA and DHA can prevent mammary tumorigenesis in DMBA model (29). We used this well-established DMBA mammary tumor model to determine if DPA can prevent or reduce the carcinogen-induced mammary tumorigenesis. Mammary carcinogenesis was initiated by a single, tail vein injection of DMBA at 7 weeks of animal age. Fifty female Sprague-Dawley rats were divided into two groups: control and DPA treated. DPA significantly reduced DMBA-induced mammary tumorigenesis (Table 1). The results reveal that DPA significantly reduced tumor incidence, the number of tumors per rat, and tumor growth. The average tumor weight (all tumors) in DPA-treated group had a mean 57% reduction when compared with tumors in control rats. Tumor incidence also reduced from 85% in control group to 52% in DPA group. The total number of tumors per rat was about 1.8-fold higher in the control group than in the DPA group. These data indicate that DPA suppresses mammary tumorigenesis in carcinogen-induced mammary tumor model.

Experimental group	Tumor incidence Tumor/Total (%)	Number of tumor per rat	Tumor weight (g)
Control	85	2.2 ± 1.8	2.8 ± 4.9
DPA	52	1.3 ± 0.8	1.2 ± 1.7

Table 1. Female Sprague-Dawley rats at 50 days of age were treated with DMBA (0.5mg/100g body weight) by a single tail vein injection in an oil emulsion. Both DPA ethyl ester (Sigma) and core oil (for control group) were administered orally at a dose of 0.5 ml/day/rat and 0.3 microg/100 microL propylene glycol twice a week respectively and continued to 30 weeks after DMBA administration. There were 25 rats analyzed for each group. Tumor growth inhibition was calculated by comparison of DPA treated vs. non-treated rats. Statistical comparison for tumor size in DPA-treated rats relative to control rats indicate $p < 0.05$. Statistical comparison for tumor incidence in DPA rats relative to control rats indicates $p < 0.05$.

ADDITIONAL NEW DATA

Introduction.

Recent studies indicate that estrogen signaling is mediated by both genomic **nuclear-initiated estrogen signaling (NIES)** (19-21) through transcriptional activation of the target genes by specific nuclear estrogen receptors designated as ERα-66, ERα-46, and ERβ and non-genomic **membrane-initiated estrogen signaling (MIES)** (22-24) effects, which is thought to be directed via plasma membrane ER, e.g., recently identified **ERα-36** (25-26). One of the MIES is the ligand-induced quick and non-genomic activation of Erk (26-27).

Preliminary studies suggest that n-3 PUFAs suppress some E2-dependent activities by inhibition of E2-activated and membrane ER-mediated MIES: Erk1 and mTOR activation. We **hypothesize** that the contribution of n-3 PUFA to inhibit hormone-dependent activities within mammary gland is mediated at least by at inhibiting MIES effect by suppressing ERα function particularly the membrane-bound ER-α36.

Results

I. Synuclein gamma stimulates membrane initiated estrogen signaling by chaperoning ER-α36, a variant of ER-α (published in American. J. Pathology, attached)

Synuclein gamma (SNCG), previously identified as a breast cancer specific gene, is highly expressed in malignant cancer cells but not in normal epithelium. The molecular targets of SNCG

during breast cancer progression have not been fully identified yet. Here we analyzed the effect of SNCG on stimulation of membrane-initiated estrogen signaling. While SNCG expression enhanced estrogen-induced activation of ERK1/2 and mTOR, knockdown of endogenous SNCG decreased membrane-initiated estrogen signaling. SNCG functions as a molecular chaperone protein for ER-α36, a membrane-based variant of ER-α. SNCG bound to ER-α36 in the presence and absence of functional molecular chaperone Hsp90. Disruption of Hsp90 with 17-AAG significantly reduced ER-α36 expression and membrane-initiated estrogen signaling. However, expression of SNCG prevented ER-α36 degradation and completely recovered 17-AAG-mediated downregulation of estrogen signaling. The function of SNCG in ER-α36-mediated estrogen signaling is consistent with its ability to stimulate cell growth in response to estrogen. Expression of SNCG also renders tamoxifen resistance, which is consistent with the clinical observation on the association of ER-α36 expression and tamoxifen resistance. The present study indicates that ER-α36 is a new member of ER-α family that mediates membrane-initiated estrogen signaling, and that SNCG can replace the function of Hsp90, chaperone ER-α36 activity, stimulate ligand-dependent cell growth, and render tamoxifen resistance.

II. Inhibition of E2-stimulated MIES by n-3 PUFA EPA. Since a higher n-6/n-3 PUFA ratio is considered to be a risk factor for breast cancer, we are interested in studying if the inhibitory effect of n-3 PUFA on breast cancer is manifested with an inhibition of MIES. Effect of n-3 PUFA on regulation of ER signaling was evaluated in ERα-negative but ERα-36 transfected MDA-MB-435 cells, which only express transfected ER-α36 but not ER-α66 and ER-α46. As demonstrated in Fig. 4A, enforced expression of ERα-36 renders E2-stimulated Erk activation. While treatment of MDA435/ER36 cells with EPA did not affect the basal levels of activated Erk, pre-treatment of cells with EPA significantly inhibited the E2-stimulated activated Erk. These data suggest that inhibition of E2-stimulated MIES is mediated, at least in part, by inhibition of ERα-36 signaling.

It has been reported that the sensitivity of breast cancer cells to mTOR inhibitor rapamycin is partially attributed to activation of the PI3K/Akt/mTOR pathway by non-genomic ER signaling. We studied if E2 stimulates mTOR activation and if n-3 PUFA inhibits the activation (Fig. 4B). Treatment of MCF-7 cells with E2 induced a rapid phosphorylation of S6K, a downstream effector of mTOR; while n-3 PUFA EPA had no effect on the basal level of phosphorylated S6K, it significantly inhibited E2-stimulated S6K activation. These data suggest that n-3 PUFA inhibits the E2-inducd and membrane ER-mediated signaling.

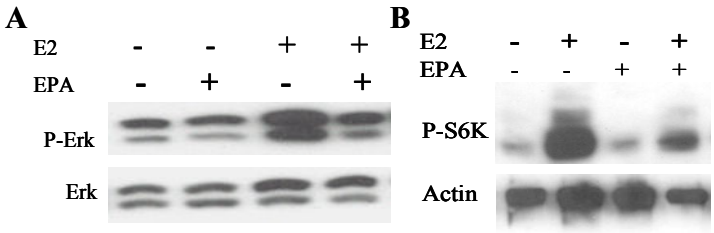


Fig. 4. A. Inhibition of E2-stimulated Erk activation by EPA. hER-α36 transfected MDA-MB-435 cells were cultured (stripped FCS, phenol red free) for 2 days in the presence or absence of 10 μM EPA and then treated with 1 nM E2 for 10 minutes. Total cell lysates were subjected to Western analyses. **B**. Inhibition of E2-stimulated S6K activation by EPA. Cells were pre-treated with or without 10 μM EPA for 24 hours, and stimulated with E2 (1 nM) for 15 minutes. Total protein was subjected to Western analyses of phosphorylated S6K and actin.

III. Differential effect on E2-mediated nuclear-initiated NIES and membrane-initiated MIES. Since EPA significantly attenuates E2-stimulated nongenomic MIES: Erk and mTOR activation (Fig. 4), we were interested in studying whether EPA has a similar effect on genomic nuclear ERα transcriptional activation. We used an *ex vivo* model involving mouse whole organ culture of mammary gland

(**OCMG**) to study effects of EPA on NIES and MIES. NIES effect was evaluated by analysis of ER-mediated transcriptional activity on E_2-regulated genes of PS2; MIES effect was evaluated by analysis of Erk activation. We first studied whether ER-α36 is present in the mouse mammary gland. Using MCF-7 cells as a positive control, Western blot revealed a specific ER-α36 band in the mouse mammary glands (**Fig. 5A**). To avoid the different *in vivo* estrogenic environments due to the different hormonal cycling of mice, virgin mice were ovariectomized and a pair of inguinal mammary glands was isolated 1 week after surgery as we previously described (28). The glands were cultured in the organ culture for 3 days and then treated with E2 in the presence or absence of EPA. In this *ex vivo* model, treatment of the glands with 2 nM E2 significantly stimulated PS2 mRNA expression resulting in an average 4.7-fold increase over control non-treated glands (**Fig. 5B**). EPA treatment slightly inhibited E2-stimulated PS2 expression causing an average 11% reduction of PS2 mRNA (**Fig. 5C**). A short (20 minutes) treatment of the glands with E2 resulted in a robust activation of Erk (**Fig. 5D**). Consistent with the observed inhibition of Erk activation by EPA in cells, pre-treatment of the glands with EPA significantly attenuated E2-induced Erk activation (**Fig. 5E**). Densitometry analysis of the intensity of the bands indicates that EPA treatment reduced phosphorylated Erk by 65% (**Fig, 5F**). These data suggest that n-3 PUFA EPA may preferentially inhibit membrane-initiated ER signaling.

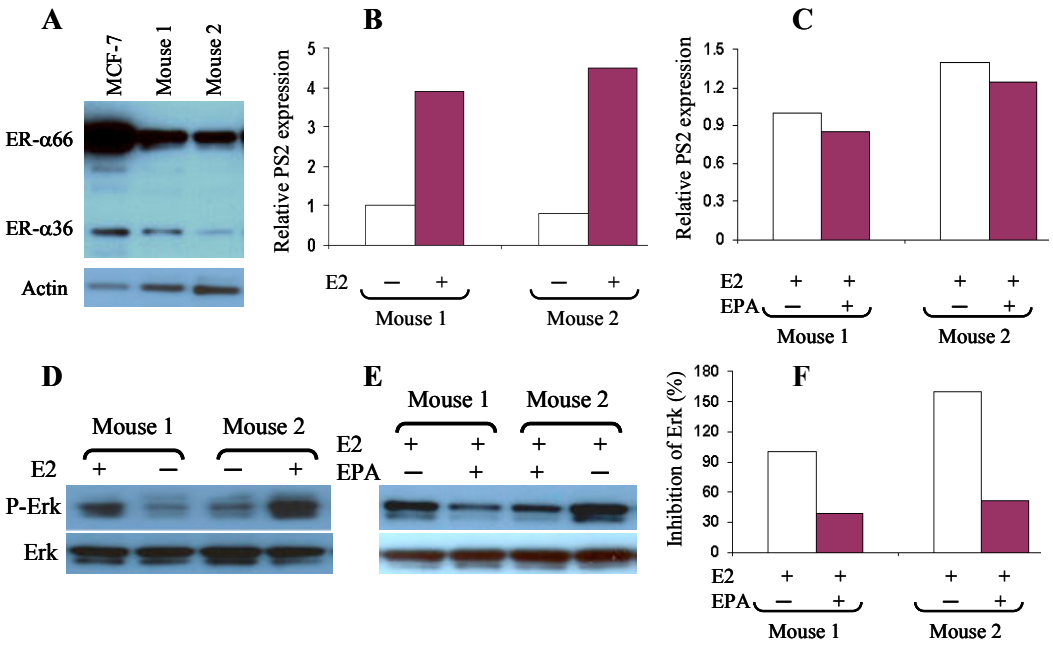


Fig. 5. A. Expression of ER-α36 in mouse mammary glands. Right inguinal whole mammary glands were removed from two 14-week virgin mice (FVB/n background). Total proteins were isolated from the glands and subjected to Western analysis of ER (anti-ERα antibody RB9016, Lab Vision) and actin. **B-C**, Regulation of PS2 expression in mouse mammary organ culture. Four pairs of inguinal whole mammary glands were removed from 14-week virgin mice 1 week following ovariectomy. The glands were cultured in the organ culture (containing 5% FCS, bovine pituitary extract 52 μg/ml, insulin 5 μg/ml, EGF 10 ng/ml, and hydrocortisone 1 μg/ml) for 3 days before the treatments. **B**. The left glands were treated with vehicle as control and the right glands were treated with 2 nM E2 for 30 hours. RNAs were isolated and subjected to real time PCR analysis using the TaqMan PCR core reagent kit (Applied Biosystems). Relative expressions of mouse PS2 gene in the E2-treated glands were calculated in comparison to that from non-treated control gland from mouse 1 (first bar), which was taken as 1 and regarded as control. All the other values were expressed as a fold of the control. The mouse beta actin gene was used as endogenous control. **C**. The glands were pretreated with or without 10 μM EPA for 18 h, followed by a 30-h treatment with 2 nM E2. Relative expressions of mouse PS2 gene in the EPA- and E2-treated glands were calculated in comparison to that from E2-treated

control gland from mouse 1 (first bar), which was taken as 1 and regarded as control. All the other values were expressed as a fold of the control. **D-E**. Western analysis of Erk activation in four pairs of inguinal mammary glands from four 14-week virgin mice. **D**. Glands were treated with or without 2 nM E2 (vehicle for left gland; E2 for right gland) for 20 min. **E**. Glands were pretreated with or without 10 μM EPA for 18 h (vehicle for left gland; EPA for right gland) followed by a 20-min treatment with 2 nM E2. **F**. Densitometry analysis of the intensity of the bands of EPA-suppressed Erk activation in **E**. E2-stimulated and non EPA treated gland from mouse 1 was taken as 100% and regarded as a control (first bar). All the other values were expressed as a percentage of the control.

KEY RESEARCH ACCOMPLISHMENTS AND REPORTABLE OUTCOMES

1. The functional role of n-3 PUFAs on differentiation was supported by the studies in fat-1 transgenic mouse, which converts endogenous n-6 to n-3 PUFAs. Alternation of n-6/n-3 ratio in favor of n-3 PUFA and particularly DPA in the mammary gland of fat-1 mouse resulted in development of lobuloalveolar-like structure and milk protein β-casein expression, mimicking differentiated state of the pregnant gland
2. DPA and EPA activated Jak2-Stat5 pathway and induced a functional differentiation with production of β-casein.
3. DPA induced mammary gland functional differentiation is dependent on Jak2-Stat5 signaling.
4. DPA treatment reduced DMBA-induced mammary carcinogenesis.
5. ER-α36 is expressed in breast cells and clinical breast cancer specimens; ER-α36 is predominantly expressed in plasma membrane. Interestingly, there is an abundant ER-α36 protein can be detected in MDA-MB-231 and MDA-MB-436 cells, two well known "ER-α-negative**"** breast cancer lines.
6. ER-α36 mediates hormone-induced non-genomic rapid MIES and cell proliferation. Transfection of ER-α36 into ER-negative MDA-MB-435 cells renders E2-induced Erk activation and cell growth. ER-α36 is a new member of ER-α family that mediates membrane-initiated estrogen signaling (attached publication).
7. n-3 PUFA EPA inhibits E2-induced Erk and mTOR activation in cells (**Fig. 4**) and Erk activation in mouse whole mammary gland in organ culture (**Fig. 5**); EPA also slightly inhibits ERα nuclear transcriptional stimulation of PS2 gene, but the such inhibition is much less significant than its inhibitory effect on membrane initiated Erk activation.
8. Publication: Shi YE, Chen Y, Dackour R, Potters L, Wang Z and Liu YE. Synuclein gamma stimulates membrane initiated estrogen signaling by chaperoning ER-α36, a variant of ER-α. **Am J Pathol**. 2010;177(2):964-73 **(**Attached).

CONCLUSIONS

The possibility of preventing breast cancer with dietary factors that induce mammary differentiation is of practical interest to the high-risk women. We studied if pregnancy-mediated breast cancer prevention is associated with an alternation of n-6/n-3 ratio in favor of n-3 PUFA. Notable findings to this study are there is a change in n-3 to n-6 PUFA composition, favoring a lower n-6/n-3 ratio in mammary gland following pregnancy and more interestingly, there is a significant increase in n-3 PUFA DPA and EPA in the pregnant mammary. Our data suggest alternation of n-6/n-3 ratio in favor of n-3 PUFA and particularly DPA and EPA may be one of the underlying mechanisms for pregnancy-mediated mammary differentiation. In supporting this novel notion, we demonstrated a similar n-6/n-3 ratio change and a differentiated phenotype in the mammary gland from the transgenic mouse expressing fat-1 gene that converts endogenous n-6 to n-3 PUFAs. In addition, the differentiation effect of DPA and EPA on mammary gland was also demonstrated in the mouse mammary organ

culture. Our studies have taken two well-established epidemiological observations and animal studies of the decreased risk of breast cancer in association with pregnancy-induced differentiation and n-3 PUFA, to point to an under-explored area mechanistically linking an alternation of n-6/n-3 ratio and particularly DPA and EPA to pregnancy-induced differentiation and potential breast cancer prevention. It is noteworthy to emphasize here that because the degree of mammary gland differentiation induced by n-3 PUFAs is not likely to be compatible to the differentiation that occurs during full term pregnancy, we are not sure whether the induced gland differentiation is one of the major contributing factors for n-3 PUFA-mediated breast cancer prevention. In addition, we also demonstrated that n-3 PUFA EPA inhibits membrane-initiated estrogen signaling by suppressing E2-stimulted Erk and mTOR activation.

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Tumorigenesis and Neoplastic Progression

Synuclein γ Stimulates Membrane-Initiated Estrogen Signaling by Chaperoning Estrogen Receptor (ER)-α36, a Variant of ER-α

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Synuclein γ (SNCG), previously identified as a breast cancer-specific gene, is highly expressed in malignant cancer cells but not in normal epithelium. The molecular targets of SNCG during breast cancer progression have not been fully identified. Here we analyzed the effect of SNCG on stimulation of membrane-initiated estrogen signaling. While SNCG expression enhanced estrogen-induced activation of ERK1/2 and mammalian target of rapamycin, knockdown of endogenous SNCG decreased membrane-initiated estrogen signaling. SNCG functions as a molecular chaperone protein for estrogen receptor (ER)-α36, a membrane-based variant of ER-α. SNCG bound to ER-α36 in the presence and absence of functional molecular chaperone heat shock protein 90. Disruption of heat shock protein 90 with 17-AAG significantly reduced ER-α36 expression and membrane-initiated estrogen signaling. However, expression of SNCG prevented ER-α36 degradation and completely recovered 17-AAG-mediated down-regulation of estrogen signaling. The function of SNCG in ER-α36-mediated estrogen signaling is consistent with its ability to stimulate cell growth in response to estrogen. Expression of SNCG also renders tamoxifen resistance, which is consistent with the clinical observation on the association of ER-α36 expression and tamoxifen resistance. The present study indicates that ER-α36 is a new member of the ER-α family that mediates membrane-initiated estrogen signaling and that SNCG can replace the function of heat shock protein 90, chaperone ER-α36 activity, stimulate ligand-dependent cell growth, and render tamoxifen resistance. ***(Am J Pathol 2010, 177:964–973; DOI: 10.2353/ajpath.2010.100061)***

Estrogen signaling is mediated by both genomic nuclear-initiated estrogen signaling by nuclear estrogen receptors (ERs) designated as ERα-66, ERα-46, and ERβ through transcriptional activation of the target genes[1–4] and non-genomic membrane-initiated estrogen signaling (MIES), which is thought to be directed via membrane-based ER. MIES was found to activate different cytoplasmic signaling proteins and other membrane-initiated signaling pathways including the adenylate cyclase,[5] the phospholipase C,[6] G protein coupled receptor-activated,[7] and the mitogen-activated protein kinase MAPK.[7–9] It was reported in the early 1970s that 17β-estradiol (E2) binds to a cell surface receptor and stimulates a rapid generation of cAMP;[10] since then evidence has accumulated to indicate a plasma membrane-based ER that transduces membrane-initiated estrogen signaling appeared.[11–13] Most recently, we reported the identification of a predominantly cell membrane-based 36-kd novel isoform of ER-α66 and designated it as ER-α36.[14–15] ER-α36 is generated from a promoter located in the first intron of the ER-α66 and lacks both ligand-independent AF-1 and ligand-dependent transcriptional AF-2 domains of ER-α66 but retains DNA-binding domain and partial ligand-binding domain. ER-α36 is predominantly on the plasma membrane, and also in cytoplasm where it transduces both estrogen- and tamoxifen-induced activation of MAPK/ERK1/2 signaling and stimulates cell growth.[15] Thus, ER-α36 plays an important role in mitogenic estrogen signaling. However, the molecular mechanisms un-

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derlying the regulation of ER-α36 function are largely unknown.

We previously identified a breast cancer specific gene BCSG1, also named as synuclein γ (SNCG).[16] Synucleins are a family of small proteins consisting of three known members, synuclein α (SNCA), synuclein β (SNCB), and SNCG.[17] While synucleins are highly expressed in neuronal cells and are abundant in presynaptic terminals, and SNCA and SNCB have been specifically implicated in neurodegenerative diseases,[18–19] SNCG is not involved in neurodegenerative diseases but primarily involved in neoplastic diseases.[16,20–24] SNCG is highly expressed in breast carcinomas and predicts poor clinical outcome in breast cancer.[24–25] When overexpressed, SNCG stimulates growth of hormone-dependent breast cancer cells both *in vitro* and in nude mice.[26–27] Expression of SNCG in mammary gland in the transgenic mice induces a highly proliferative pregnancy-like phenotype of mammary epithelial cells and the gland hyperplasia.[28] Investigations aimed to elucidate the molecular mechanisms underlying the oncogenic functions of this protein reveal that expression of SNCG in cancer cells results in a more malignant phenotype with increased cell motility,[20] enhanced transcriptional activity of steroid receptors,[26–28] and accelerated rate of chromosomal instability.[29–30] The contribution of SNCG to breast cancer development and progression may be due to its chaperone activity on both estrogen (E2)-dependent and E2-independent pathways. Previously, we demonstrated that SNCG participates in the heat shock protein 90 (Hsp90)-based multichaperone complex for steroid receptors and stimulates ER-α66 transcriptional activity but does not affect ER-β signaling.[26–27] The present study demonstrated SNCG as a tumor specific chaperone, which can replace the chaperoning function of Hsp90 and protect and stimulate ER-α36-mediated MIES.

Materials and Methods

Reagents

Antibodies used for immunoprecipitation and Western blot analyses were as follows: anti-γ-synuclein antibody (goat polyclonal antibody E-20); anti-ER-α antibody (rabbit polyclonal antibody HC-20); a specific peptide anti-ER-α36 antibody,[14–15] anti-Hsp90 antibody (rabbit polyclonal antibody sc-7947); normal goat IgG (sc-2028); and anti-actin antibody (goat polyclonal antibody sc-1615). These antibodies were from Santa Cruz Biotechnology. Anti-ERK1/21/2, anti-phospho-ERK1/21/2, anti-S6K, and anti-phospho-S6K were from Cell Signaling Technology (Beverly, MA).

Cell Culture

Proliferating subconfluent human breast cancer cells were harvested and cultured in the steroid-stripped condition (phenol red-free IMEM containing 5% dextran-charcoal-stripped fetal calf serum) for 3 days before addition of indicated dose of E2.

Development of Antibody Specific to ER-α36

We developed affinity-purified rabbit polyclonal anti-ER-α36 antibodies (Abs) as a custom service from Alpha Diagnostic (San Antonio, TX). The Abs were raised against a synthetic peptide antigen corresponding to the unique C-terminal 20 amino acids of human ER-α36. The specificity of the Ab was tested in ER-α36 expression vector transfected HEK293 cells that do not express endogenous ER-α.[15]

Cell Proliferation Assay

Cell growth was measured using a cell proliferation kit (XTT; Roche Molecular Biochemicals, Germany).

Transfection of SNCG into MCF-7 and MDA-MB-435 Cells

SNCG stably transfected MCFB6 and SNCG-435-3 cells were previously established and described.[26,20]

Stable Expression of SNCG Antisense mRNA in T47D Cells

Knockdown endogenous SNCG expression in T47D cells was achieved by antisense approach as described previously.[26] Briefly, a 285-bp DNA fragment corresponding to the exon 1 region (−169 to +116) of SNCG gene was amplified from the plasmid pBS-SNCG759 and was cloned into the EcoRI site of the expression vector pcDNA3.1.Vectors expressing SNCG antisense mRNA (pcDNA-SNCG-As) or SNCG sense mRNA (pcDNA-SNCG-S) were separately transfected into T47D cells by Effectin reagent. Two stably SNCG antisense transfected clones (AS-3 and AS-1) were selected and characterized.[26]

Knockdown of Endogenous SNCG in MDA-MB-231 Cells by RNA Interference

To knock down endogenous SNCG, we used SNCG siRNA lentiviral particle gene silencers (42290-v from Santa Cruz). SNCG siRNA consists of a pool of five target-specific 19- to 25-nucleotide siRNAs designed to knock down SNCG gene expression. Subconfluent MDA-MB-231 cells were infected with the lentiviral vectors according to the manufacturer's protocol.

In Vitro *Pulldown Assay using Glutathione-S-Transferase*

The complete coding region of SNCG was inserted into pGEX-5X-1 (Amersham Bioscience). For the glutathione-*S*-transferase (GST) pulldown assay, full-length SNCG

fused to GST (GST-SNCG) or GST alone was expressed in *Escherichia coli* BL21, which was then purified on glutathione-Sepharose 4B beads (Amersham Pharmacia). MCF-7 and ER-α36-transfected MDA-MB-435 cells (5×10^6 cells/dish) were rinsed three times with 1 ml of ice-cold PBS and sonicated in 1 ml of lysis buffer (20 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 50 mmol/L NaF, and 1 mmol/L Na_3VO_4, containing protease inhibitors). Cell lysates were spun at 12,000 $\times$ *g* for 10 minutes at 4°C to remove debris. Supernatants were incubated for 12 hours at 4°C with GST or GST-SNCG bound to beads. The beads were then washed three times in lysis buffer. Proteins were subjected to Western analysis of ER-α36 with a specific peptide antibody.

Immunoprecipitation (IP)

IP was performed as previously described.[27]

Soft Agar Colony Formation Assay

The anchorage-independent growth was performed in 12-well plates as previously described.[26]

Tumor Growth in Athymic Nude Mice

A nude mouse tumorigenic assay was performed as we previously described.[27] Briefly, 17β-estradiol pellets (0.72 mg/pellet, 60-day releasing, Innovative Research of America, Toledo, OH) were implanted subcutaneously in athymic nude mice (Frederick Cancer Research and Development Center, Frederick, MD) 1 day before the injection of hormone-dependent MCF-7 and MCFB6 tumor cells. Approximately 3×10^6 cells were injected into a 6-week-old female athymic nude mouse. When tumor xenografts were established, mice bearing tumors were randomly allocated to different treatment groups. Tamoxifen treatment was given subcutaneously 3 days/week. Each animal received two injections, one on each side, in the mammary fat pads between the first and second nipples. Tumor size was determined every 10 days by three-dimensional measurements (in millimeters) using a caliper. Only measurable tumors were used to calculate the mean tumor volume for each tumor cell clone at each time point.

Statistical Analysis

Results were reported as the mean $\pm$ SD for typical experiments done in three replicate samples and compared by the Student's *t*-test. Results were considered significantly different for $P < 0.05$. All experiments were done at least twice to ensure reproducibility of the results.

Results

Expression of ER-α36 in Breast Cancer Cells

We first conducted Western blot analysis on ER-α36 expression in ER-positive breast cancer T47D and MCF-7 cells, ER-negative MDA-MB-435, MDA-MB-231, MDA-MB-436 cells, and HT29 colon cancer cells as a control for non-breast cells. Figure 1A demonstrates that while ER-α36 is not expressed in ER-negative MDA-MB-435 cells, it is expressed in both MDA-MB-231 and MDA-MB-436 cells, two well-known ER-α66-negative breast cancer cell lines. ER-α36 is also expressed in ER-positive breast cancer T47D and MCF-7 cells. ER-α36 expression was not detected in HT29 colon cancer cells. These data are consistent with previous publication on ER-α36 expression in both ER-negative and-positive breast cancer cells.[15] We transfected ER-negative MDA-MB-435 cells with hER-α36 expression vector. This transfection showed an abundant ER-α36 expression in MDA-MB-435/ER36 cells. To determine whether ER-α36 is subjected to hormonal regulation, expression of ER-α66 and ER-α36 in MCF-7 and T47D cells was analyzed (Figure 1B). While limited amount of ER-α36 was observed when both cell lines were cultured in the absence of estrogen, treatment of the cells with 17β-estradiol (E2) and tamoxifen induced a significant increase in expression of ER-α36. As expected, both E2 and tamoxifen treatment resulted in a down-regulation of ER-α66.

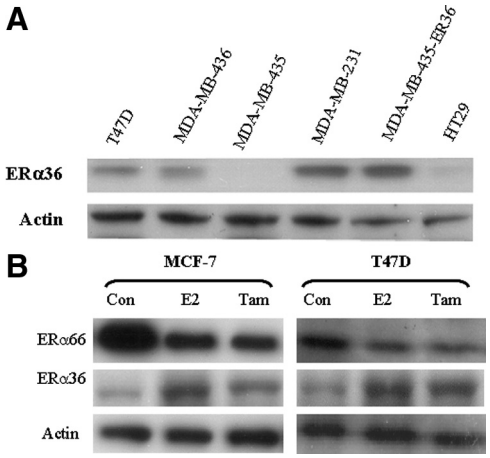


Figure 1. Expression of ER-α36. **A:** Expression of ER-α36 in different cells. Western blot analysis of cell extracts with an ERα36-specific Ab generated against the C terminus of the human ER-α36 protein.[15] For transfection, MDA-MB-435 cells were transiently transfected with hERα-36 plasmid pCI-ER36 or the control vector pCI-neo and then selected with G418. **B:** Regulation of ER-α36 expression by E2 and tamoxifen. MCF-7 and T47D cells were cultured in 5% stripped fetal calf serum (phenol red free IMEM) for three days and then treated with 2 nmol/L E2 or 1 μmol/L tamoxifen for two days. Total cell lysates were isolated and subjected to Western analyses with anti-ERα antibody (RB9016, Lab Vision) and specific peptide antibody against ER-α36.

Enhancement of Membrane-Initiated Estrogen Signaling by SNCG

We previously demonstrated that SNCG expression in MCF-7 cells significantly enhanced transactivation of ER-α66.[26–28] We decided to test whether SNCG also regulates MIES. We examined ERK1/2 activation in SNCG-negative MCF-7 cells and previously established SNCG stably transfected MCFB6 cells[26–27] in the absence and presence of E2 or tamoxifen. In contrast to genomic

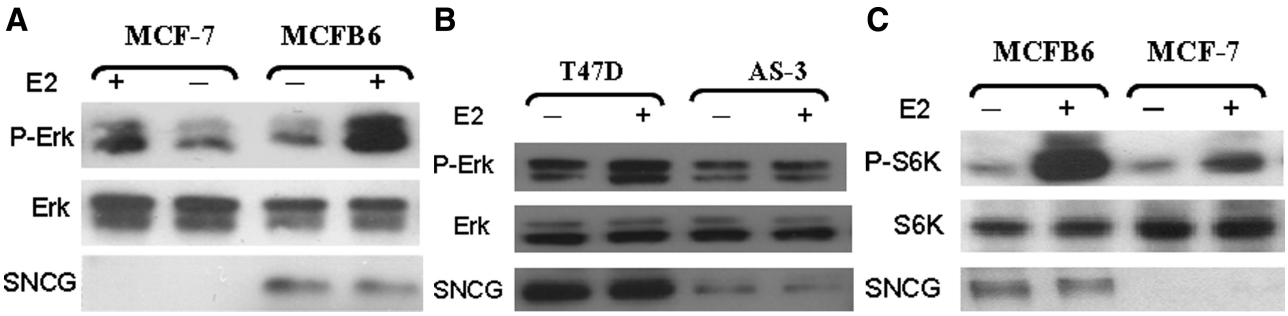


Figure 2. Stimulation of membrane ER-mediated ERK1/2 and mTOR activation by SNCG. **A:** MCF-7 and its SNCG stably transfected clone MCFB6 cells are cultured in the stripped condition. Cells were treated with 2 nmol/L E2 for 15 minutes. Total cell lysates were subjected to Western analyses of ERK1/2, phosphorylated ERK1/2, and SNCG. **B:** T47D and its SNCG knockdown AS-3 cells. Cells were cultured and treated as described for MCF-7 and MCFB6 cells. **C:** Enhancement of E2-induced mTOR signaling. MCF-7 and SNCG stably transfected MCFB6 cells were cultured in the stripped condition. Cells were treated with or without E2 (2 nmol/L) for 15 minutes. Total protein was subjected to Western analyses of S6K, phosphorylated S6K, and SNCG. Comparing the intensity of bands on a Western blot was determined by densitometry.

nuclear-initiated estrogen signaling, which requires a longer time treatment; hormone-induced MIES is rapid and occurs several minutes after the treatment. As shown in Figure 2A, treatment of MCF-7 cells with E2 for 15 minutes induced a rapid 2.9-fold phosphorylation of ERK1/2. The magnitude of hormone-induced ERK1/2 activation was significantly enhanced in MCFB6 cells; E2 treatments resulted in a 6.2-fold phosphorylation of ERK1/2 over control cells. The effect of SNCG on E2-stimulated MIES was further demonstrated in the previously established AS-3 cells derived from T47D cells with stable SNCG knockdown, which reduced SNCG expression to 15% of that of control T47D cells.[26] Whereas E2 strongly stimulated ERK1/2 activation in control T47D cells that express endogenous SNCG, resulting in a 5.8-fold ERK1/2 phosphorylation, knockdown of SNCG expression significantly reduced E2-stimulated ERK1/2 activation with only a 2.2-fold increase (Figure 2B).

A growing body of evidence has suggested that mammalian target of rapamycin (mTOR) acts as a sensor that integrates extracellular and intracellular events, coordinating cell proliferation and survival.[31] Rapamycin, an inhibitor of mTOR, possesses antitumor activity against many tumors including breast tumors, and particularly against ER-positive breast cancer cell lines. The sensitivity of these cells to rapamycin has been attributed to activation of the PI3K/Akt/mTOR pathway by nongenomic ER signaling.[32–34] We tested if SNCG expression affects E2-stimulated mTOR activation (Figure 2C). Treatment of MCF-7 cells with E2 stimulates the phosphorylation of S6K, a downstream effector of mTOR. However, an E2-stimulated S6K phosphorylation in MCFB6 cells stably transfected a SNCG expression vector was significantly enhanced versus control MCF-7 cell. The data suggest that SNCG enhances the E2-inducerd rapid activation of the mTOR signaling. Taken together, the increased E2-stimulated ERK1/2 and mTOR activation in SNCG-transfected MCF-7 cells and the down-regulated ERK1/2 activation in SNCG knockdown T47D cells indicated that SNCG enhances ligand-dependent MIES.

SNCG Stimulates ER-α36-Mediated ERK1/2 Activation

While alternation of SNCG expression in MCF-7 and T47D cells affects MIES, it is not clear which ER-α isoform mediates this effect. Furthermore, the E2-induced activation of non-genomic MIES in MCF-7 and T47D cells could also be mediated by GPR30, a G protein-coupled receptor for estrogen.[35] In an effort to dissect the contributions of different receptors—ER-α66, ER-α46, ER-α36, and GPR30—to observed SNCG activity in E2-stimulated ERK1/2 activation, and to determine whether ER-α36 can mediate E2-induced MIES, we studied ERK1/2 activation in ER-α-negative MDA-MB-435 and ER-α36 transfected MDA-MB-435/ER36 cells. As shown in Figure 3A, while treatment MDA-MB-435 cells with E2 did not stimulate activation, enforced expression of ER-α36 in MDA-MB-435 cells rendered ERK1/2 activation in response to E2, resulting in a 3.1-fold increase in ERK1/2 activation. The ER-α36-stimulated stimulation of ERK1/2 activation was ligand-dependent because ER-α36 had no significant effect on the ERK1/2 activation in the absence of E2. We next co-transfected MDA-MB-435 cells with SNCG and ER-α36 and studied the effect of SNCG on ER-α36-mediated ERK1/2 activation by E2. A significant stimulation of E2-induced ERK1/2 activation by SNCG was observed in MDA-MB-435 cells co-transfected with ER-α36 and SNCG expression constructs. While SNCG had no effect on the basal levels of the ERK1/2 activation, SNCG increased ligand-dependent ERK1/2 activation twofold over the control SNCG-negative cells.

Although there has been a controversy over the past several years about the true origin of the human MDA-MB-435 cell line, which might be derived from M14 melanoma cells, recent evidence suggests that the idea that the MDA-MB-435 cell line indeed represents a poorly differentiated, aggressive breast tumor line, with expression of both epithelial and melanocytic markers, should be reconsidered.[36] We also used ER-negative (ER-α66-negative) but ER-α36- and SNCG-positive MDA-MB-231 breast cancer cells to study the effect of SNCG on ER-α36-mediated MIES. The effect of SNCG expression on

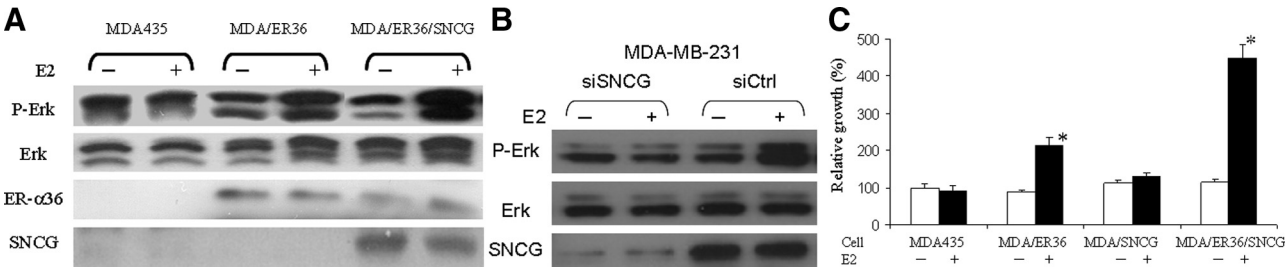


Figure 3. SNCG stimulates ER-α36 signaling. **A:** Activation of ERK1/2 in ER-α36-transfected MDA-MB-435 cells. Cells were co-transfected with ER-α36 and pCI-SNCG or ER-α36 and the control vector pCI-neo and then selected with G418. Cells were cultured in stripped condition for two days and then treated with or without 2 nmol/L E2 for 15 minutes. Total cell lysates were isolated and subjected to Western analyses with antibodies against phosphorylated ERK1/2, total ERK1/2, and ERα−36. **B:** Effect of knockdown of endogenous SNCG on ERK1/2 activation on MDA-MB-231 cells. Control siRNA (siCtrl) and SNCG siRNA infected MDA-MB-231 cells were treated with 2 nmol/L E2 for 15 minutes. Phosphorylated ERK1/2, ERK1/2, and SNCG were analyzed by Western blot. Comparison of the intensity of bands on a Western blot was determined by densitometry. **C:** SNCG stimulated ligand-dependent and ER-α36-mediated cell proliferation. Control MDA-MB-435, ER-α36 transfected MDA/ER36, SNCG transfected MDA/SNCG, and ER-α36/SNCG double transfected MDA/ER36/SNCG cells were cultured in the stripped condition for three days before the hormone treatments. Cells were treated with or without 1 nmol/L E2 for four days. Cell proliferation was measured by using a cell proliferation kit (XTT). The numbers represent means ± SD of three cultures. The open bar represents non-treated cells; closed bar represents E2-treated cells. All values were presented as the percentage of stimulation over the control non-treated cells, which were taken as 100%. The **asterisk** indicates a statistical comparison of $P < 0.001$. Statistical comparisons for E2-treated ER-α36/SNCG double transfected cells relative to E2-treated ER-α36 single transfected cells indicate $^*P < 0.001$.

ER-α36-mediated MIES was demonstrated by inhibiting endogenous SNCG expression in MDA-MB-231 cells (Figure 3B). SNCG siRNA significantly reduced endogenous SNCG expression in MDA-MB-231 cells. Knockdown SNCG significantly reduced E2-stimulated ERK1/2 activation. Treatment of control siRNA-infected cells with E2 induced a 4.3-fold increase in ERK1/2 activation; the E2-induced ERK1/2 activation was greatly reduced to 1.5-fold in the SNCG knockdown cells. These data suggest that membrane-initiated estrogen signaling is mediated, at least in part, by ER-α36, and SNCG elevated ER-α36-mediated signaling.

ER-α36 and SNCG Synergistically Mediates Estrogen-Stimulated Cell Proliferation

To pursue the significance of MIES through ER-α36, we tested whether ER-α36 can mediate E2-stimulated cell proliferation. Data in Figure 3C show that E2 stimulated about twofold proliferation of ER-α36-transfected MDA-MB-435 cells compared with cells treated with vehicle. This ER-α36-mediated and E2-stimulated cell proliferation was also previously observed in HEK 293 cells.[15] These results indicated that ER-α36 acts as a functional receptor to mediate mitogenic estrogen signaling. To determine the biological significance of SNCG in the mitogenic estrogen signaling mediated by ER-α36, we analyzed the effect of SNCG expression on E2-stimulated growth in ER-α36 transfected cells. To determine whether SNCG overexpression affects ligand-dependent and ligand-independent cell growth, the cellular proliferation of ER-α36 and SNCG co-transfected MDA-MB-435 cells were compared with that of control and cells transfected with ER-α36 or SNCG expression vector individually. SNCG had no significant effect on the proliferation of SNCG-transfected cells compared with control cells in the absence of E2, which was consistent with the previous observations that SNCG only stimulates hormone-dependent growth of breast cancer cells both *in vitro* and in nude mice.[16,27] Overexpression of SNCG significantly stimulated the ligand-dependent and ER-α36-mediated proliferation. Treatment of MDA435/ERα36 cells with E2 stimulated cell proliferation 2.2-fold over controls. However, E2 treatment of MDA435/ERα36/SNCG cells resulted in a 4.5-fold increase in the proliferation versus controls. These data suggest that SNCG renders cells more responsively to E2-stimulated and ER-α36-mediated cell proliferation.

SNCG Chaperones ER-α36 and Prevents Hsp90 Inhibition Induced Down-Regulation of MIES

We previously demonstrated that SNCG physically binds to unliganded ER-α66 complex and chaperones ER-α66 transcriptional activity.[27] Since these studies were performed in the absence of E2 and we demonstrated that E2 treatment stimulates ER-α36 expression (Figure 1B), we are interested in studying if SNCG is physically associated with ER-α36 in the presence of E2. As shown in Figure 4A, in the absence of ligand, IP SNCG efficiently co-precipitated ER-α66 and small amount of ER-α36 (control). In the presence of E2, however, SNCG dissociated from the ER-α66 complex and interacted with ER-α36. These data suggest that SNCG binds to unliganded ER-α66 in the absence of E2; while SNCG dissociates from ER-α66 complex and binds to ER-α36 in the presence of E2. To further confirm this interaction, we then investigated if SNCG interacts with ER-α36 *in vitro* in the cell free system. We performed a GST pulldown assay using the purified GST-tagged SNCG protein to pull down ER-α36 (Figure 4B). The GST-tagged SNCG was immobilized to GST beads and incubated with lysates of MCF-7 and ER-α36-transfected MDA-MB-435 cells. The eluted proteins were subjected to immunoblot analysis using specific anti-ER-α36 antibody. The results of immunoblotting revealed that ER-α36 was specifically precipitated by immobilized GST-SNCG, indicating that ER-α36 directly interacts with SNCG *in vitro*.

Hsp90 is a molecular chaperone whose association is required for the stability and function of multiple signaling proteins that promote the growth and/or survival of can-

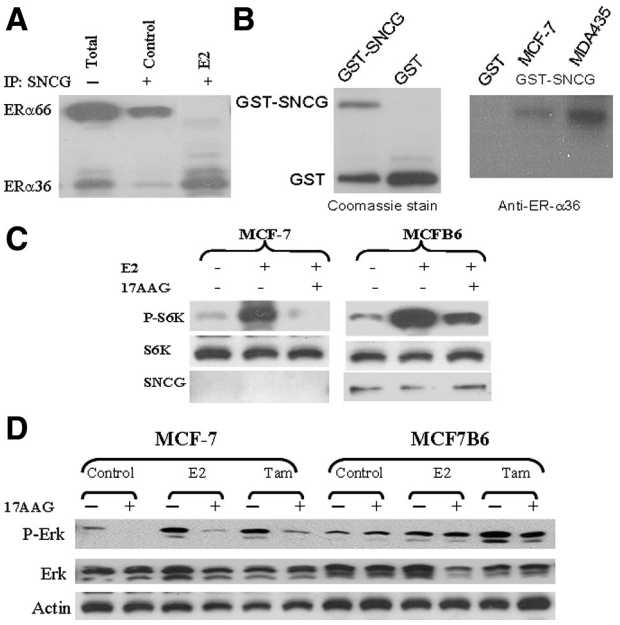


Figure 4. Prevention of loss of ER-α36 signaling by SNCG. **A:** Physical interaction between SNCG and ERα. SNCG stably transfected MCFB6 cells cultured in the stripped condition, were treated with or without 1 nmol/L E2 for 16-hour. Equal amount of protein was subjected to immunoprecipitation with anti-SNCG and followed by Western analysis of ERα. Total represents the total protein from the E2-treated cells before immunoprecipitation. **B:** GST-SNCG fusion protein was expressed in *E. coli*, purified, and stained with Coomassie Blue to demonstrate the expression of the fusion proteins (**left**). Cell extracts from MCF-7 and ER-α36-transfected MDA-MB-435 cells were subsequently incubated either with bead-bound GST as a negative control or GST-SNCG. After the beads were washed, proteins were subjected to Western blot for ER-α36 using the specific anti-ER-α36 peptide antibody (**right**). **C:** Preventing Hsp90 inhibition-induced down-regulation of mTOR signaling in MCF-7 cells by SNCG. MCF-7 and MCFB6 cells were treated with or without 1 μmol/L 17-AAG for 15 hours and stimulated with E2 (2 nmol/L) for 15 minutes. Total protein was subjected to Western analyses of phosphorylated S6K, S6K, and SNCG. **D:** Preventing Hsp90 inhibition-induced down-regulation of ERK1/2 activation in MCF-7 cells by SNCG. MCF-7 and MCFB6 cells were treated with or without 1 μmol/L 17-AAG for 15 hours and stimulated with E2 (2 nmol/L) or tamoxifen (1 μmol/L) for 15 minutes. Total protein was subjected to Western analyses of phosphorylated ERK1/2, ERK1/2, and normalized with actin.

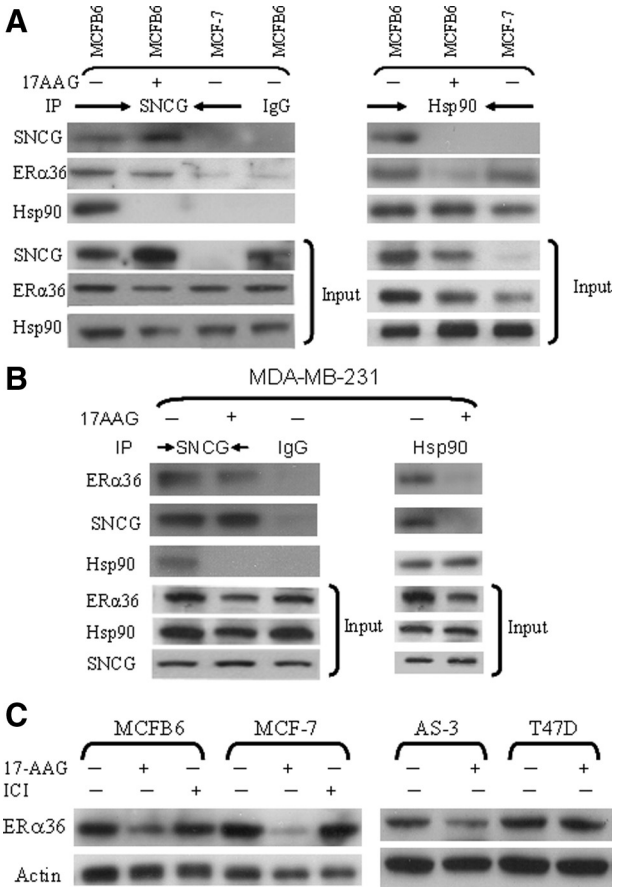
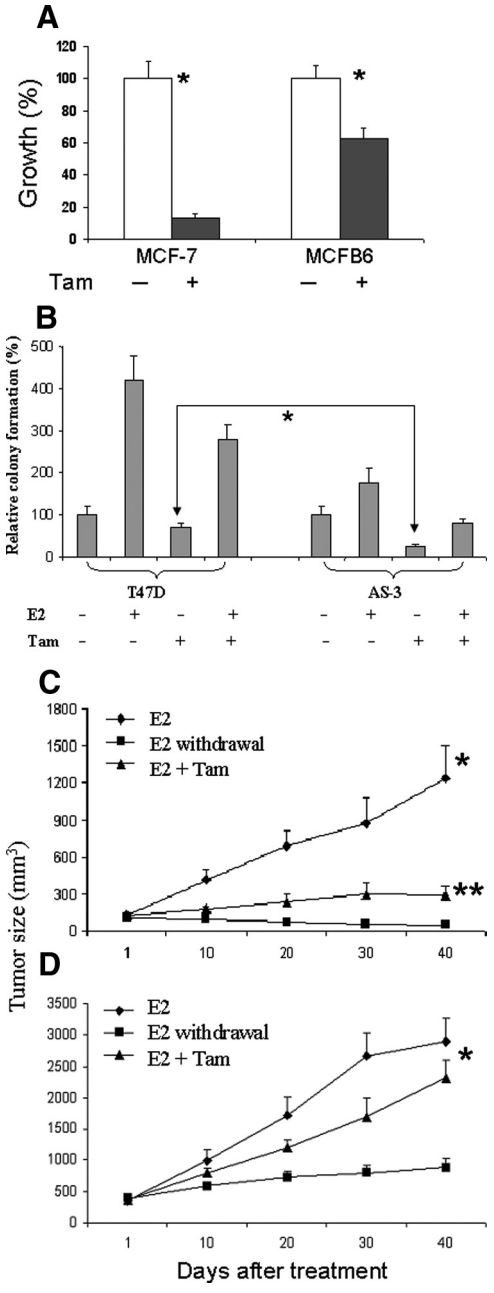


Figure 5. SNCG chaperones ER-α36. **A** and **B:** Physical interactions among SNCG, ER-α36, and Hsp90 in the presence and absence of 17-AAG. **A:** MCF-7 and its SNCG-transfected MCFB6 cells. **B:** MDA-MB-231 cells. Cells were treated with or without 1 μmol/L 17-AAG for 15 hours. Equal amount of protein was subjected to IP with SNCG, control IgG, and Hsp90 followed by Western blot for SNCG, ER-α36, and Hsp90. **C:** Prevention of ER-α36 degradation by SNCG. MCF-7, MCFB6 cells, T47D, and its SNCG knockdown AS-3 cells were treated either with 17-AAG (1 μmol/L, 12 hours) or ICI 182,780 (10 nmol/L, four hours). ER-α36 expression was analyzed by Western blot and normalized with actin.

cer cells. It is well documented that the activation of steroid receptors including ER-α is modulated by an Hsp90-based multiple chaperone complex.[37–38] Inhibition of Hsp90 by small molecules, eg, 17-AAG, leads to degradation of Hsp90 client proteins and inactivation of the corresponding signaling pathways.[39] The chaperone activity of SNCG on regulation of transcriptional activation of ER-α66 has been demonstrated in breast cancer cells[27] as well as in mammary glands.[28] Since SNCG enhances ER-α36-mediated MIES and physically interacts with ER-α36 in the presence of estrogen, we reason that SNCG may also chaperone ER-α36-mediated MIES. To determine whether disrupting Hsp90 function with 17-AAG could attenuate MIES and if SNCG expression can restore MIES activity by chaperone ER-α36, we disrupted Hsp90 function by treating the cells with 17-AAG. We demonstrated that under stressful conditions when the chaperone function of Hsp90 was blocked by 17-AAG, the loss of hormone-induced MIES was protected by SNCG. As shown in Figure 4C, while treatment of parental MCF-7 cells with 17-AAG abolished E2-induced mTOR activation as measured by the absence of phosphorylated S6K, expression of SNCG in MCFB6 cells rendered a resistance to the inactivation of mTOR due to Hsp90 disruption. We also studied the chaperone effect of SNCG on protection of ERK1/2 signaling. Both estrogen and tamoxifen treatment stimulated ERK1/2 activation in MCF-7 and MCFB6 cell (Figure 4D). Treatment of MCF-7 cells with 17-AAG resulted in a significant decrease in activated ERK1/2 in both control and hormone-stimulated cells. However, forced expression of SNCG completely restored ERK1/2 activation in MCFB6 cells. These data suggest an important role of SNCG in regulation of ER-α36-mediated MIES, which can replace the chaperone activity of Hsp90 and maintain MIES.

Using MCF-7 as well as MCFB6 cells, we determined the physical interaction among SNCG, Hsp90, and ER-α36 in the presence and absence of 17-AAG (Figure 5A). IP of SNCG in SNCG-positive MCFB6 cells brought down Hsp90 and ER-α36 in the absence of 17-AAG, indicating that SNCG participated in a chaperone complex with Hsp90 and ER-α36 in the absence of Hsp90 inhibitor. As negative controls, control IgG did not pull down SNCG,

ER-α36, and Hsp90 in MCFB6 cells; IP of SNCG in SNCG-negative MCF-7 cells did not bring down ER-α36 and Hsp90. Similarly, IP of Hsp90 co-precipitated SNCG and ER-α36 in MCFB6 cells and ER-α36 in MCF-7 cells. As expected, after cells were treated with Hsp90 inhibitor 17-AAG, Hsp90 dissociated from its client protein ER-α36. However, although SNCG dissociated from Hsp90 after the treatment with 17-AAG, it was still able to interact with ER-α36. Our data indicate that although SNCG participates in the chaperone complex with Hsp90, its function on ER-α36 is mediated by Hsp90-independent pathways such as by direct binding to and chaperoning ER-α36. We also investigated the interactions among endogenous SNCG, Hsp90, and ER-α36 in MDA-MB-231 cells (Figure 5B). The same interaction pattern among SNCG, ER-α36, and Hsp90 was observed in MDA-MB-231 cells as that we demonstrated in SNCG-transfected MCF-7 cells. When MDA-MB-231 cells were cultured in the absence of 17-AAG, endogenous SNCG was co-precipitated with Hsp90 and ER-α36. However, after the treatment of 17-AAG, Hsp90 dissociated from ER-α36 and SNCG, but SNCG still bound to ER-α36. Since SNCG is physically associated with ER-α36 at the stressful condition when the function of Hsp90 is disrupted, we reason that SNCG protects its client protein ER-α36 and prevents its degradation due to the loss of Hsp90 function. As demonstrated in Figure 5C, treatment of SNCG-negative MCF-7 cells with 17-AAG resulted in a significant loss of ER-α36; expression of SNCG in MCFB6 cells prevented this Hsp90 disruption-induced degradation of ER-α36. As a negative control, treatment of the cells with antiestrogen ICI 182,780 had no effect on the expression of ER-α36. Similar to MCF-7 cells, while SNCG-positive T47D cells were resistant to Hsp90 disruption; knockdown SNCG expression in AS-3 cells renders the reduction of ER-α36 in response to 17-AAG treatment. Taken together, these data suggest that at the stressful condition when the function of Hsp90 is disrupted, SNCG functions as a chaperone protein, which physically binds to ER-α36 and protects its expression and function.



SNCG Renders a Resistance to Tamoxifen

Recently, an association between ER-α36 expression and tamoxifen resistance was analyzed in 709 breast cancer patients with a median follow-up of 7.9 years.[40] In these studies, ER-α36 expression in ER-α66-positive tumors who received tamoxifen treatment (n = 306) was associated with poorer survival. These studies indicate that ER-α36 is an important predictive marker for tamoxifen resistance in ER-α66-positive breast cancer patients. Since SNCG chaperones ER-α36 expression and func-

Figure 6. SNCG renders tamoxifen resistance. **A:** MCF-7 and MCFB6 cells were treated with 10 μmol/L tamoxifen for four days. Media were changed every two days with fresh added tamoxifen. Cell growth was measured using a cell proliferation kit (XTT). All values were presented as the percentage reduction over the control non-treated cells, which was taken as 100%. The numbers represent means ± SD of three cultures. Comparisons for cell growth in control relative to that of tamoxifen treatment indicate $^*P < 0.001$. **B:** Colony formation of T47D and its SNCG knockdown AS-3 cells. Cells were treated with or without 1 nmol/L E2, 1 μmol/L tamoxifen, or combination of E2 and tamoxifen. The number of colonies was counted after two weeks of plating. Statistical comparison of colony formation for tamoxifen-treated AS-3 cells relative to tamoxifen-treated T47D cells indicates $P < 0.01$. **C** and **D:** Effect of tamoxifen on the growth of MCF-7 and MCFB6 xenografts. MCF-7 (**C**) and MCFB6 (**D**) tumor cell injection and estrogen supplement were described in *Materials and Methods*. There were eight mice in each group and each mouse received two injections, one on each side. Twelve days after injection, xenografts were established and tumors reached to >100 mm^3. At this stage (day 1), mice bearing established tumors were randomly allocated to three groups: group 1, continued E2; group 2, E2 withdrawal (removal of the E2 pellet); group 3, E2 plus tamoxifen treatment (500 μg/mouse given subcutaneously, three days/week). Tumor size was determined by three-dimensional measurements (mm) using a caliper. All mice were sacrificed at day 40 after the first treatment. Each point represents the mean of tumors ± SE In MCF-7 xenograft, statistical comparison for tumor size in tamoxifen treated mice relative to control (E2 group) mice indicates $^*P < 0.001$; and comparison of tamoxifen versus E2 withdrawal indicates $^{**}P < 0.05$. In MCFB6 xenograft, comparisons for tumor size in both control (E2 group) and tamoxifen treated mice relative to that of E2 withdrawal mice indicate $^*P < 0.001$; and comparisons for tamoxifen versus control indicate $P > 0.05$.

tion, we reason that SNCG may render tamoxifen resistance. As expected, treatment of MCF-7 cells with tamoxifen inhibited cell growth resulted in 90% fewer cells (Figure 6A). In MCFB6 cells, by contrast, the magnitude of tamoxifen-mediated growth inhibition was significantly attenuated, with only 30% growth inhibition achieved. The effect of SNCG expression on tamoxifen resistance was further demonstrated by inhibiting endogenous SNCG expression in T47D cells. In an anchorage-independent growth (Figure 6B), estrogen stimulated a 4.2-fold colony formation over control in SNCG expressing T47D cells, knocking down endogenous SNCG significantly reduced colony formation in AS-3 cells in response to E2, resulting in only 1.6-fold increase. While tamoxifen alone only slightly decreased colony formation in T47D cells, resulting in a 25% inhibition, a robust 74% reduction of colony formation was observed in AS-3 cells, indicating that SNCG expression renders a cell more resistance to tamoxifen.

The biological relevance of SNCG-mediated tamoxifen resistance was also investigated in an orthotopic nude mouse model. To establish tumor xenografts, all mice were supplemented with E2 1 day before injection of tumor cells. Mice bearing established tumors were then randomized into three groups: group 1, continued treatment with E2; group 2, E2 withdrawal; and group 3, E2 with tamoxifen. The growth of MCFB6 tumor was stimulated much more by E2 than parental MCF-7 tumor. At 40 days, E2-stimulated MCF-7 tumor was 1.23 cm^3 (Figure 6C) compared with that of 2.90 cm^3 for MCFB6 tumor (Figure 6D). As expected, the growth MCF-7 xenograft was significantly inhibited by tamoxifen. At 40 days following treatment, tamoxifen inhibited E2-stimulated tumor growth by 77%. Although the tumor growth of MCFB6 cells was also inhibited by tamoxifen, the magnitude of growth inhibition reduced with a slight 19% growth inhibition. E2 withdrawal completely suppressed tumor growth of MCF-7 xenograft, but MCFB6 xenograft continued to grow at very slow path in the absence of E2 supplement. Consistent with *in vitro* data, these data indicate that SNCG renders tumor tamoxifen resistance.

Discussion

The present study showed that ER-α36 is an important and new player in MIES and that SNCG positively regulates ER-α36-mediated MIES through chaperoning ER-α36 protein. While SNCG overexpression enhanced ligand-dependent MIES, compromising endogenous SNCG expression comprised hormone stimulated MIES. The SNCG-enhanced MIES was demonstrated in four different cell systems including overexpression of SNCG in SNCG-negative MCF-7 cells; knockdown endogenous SNCG expression in SNCG-positive T47D cells; overexpression of SNCG in ER-negative but ER-α36 transfected MDA-MB-435 cells; and knockdown endogenous SNCG expression in ER-α36- and SNCG-positive MDA-MB-231 cells. We provided evidence suggesting that SNCG is a new member of molecular chaperone protein, which protects and stimulates ER-α36 mediated MIES. This evidence includes that SNCG binds to ER-α36 both *in vitro* in cell-free system and in breast cancer cells; SNCG is able to interact with ER-α36 even in the presence of 17-AAG, in which Hsp90 dissociates from its client protein ER-α; SNCG prevents Hsp90 disruption-induced degradation of ER-α36; SNCG can restore Hsp90 disruption-induced down-regulation of MIES; and SNCG significantly stimulated the mitogenic estrogen signaling mediated by ER-α36. These data suggest a critical role of SNCG in maintaining the stability and function of ER-α36, and thus rendering estrogen membrane-initiated signaling. In fact, it had been demonstrated that expression of SNCG enhances E2-stimulated mammary gland proliferation in transgenic mice[28] and the growth of hormone-dependent breast cancer xenografts.[27]

Previously, we found that ER-α36 strongly attenuates the transactivational activity mediated by both AF1 and AF2 domains of ER-α66,[15] indicating that ER-α36 negatively regulated genomic estrogen signaling. Recently, we also found the ER-α66 negatively regulates the promoter activity of the ER-α36 gene.[41] Taken together, these results indicated that ER-α isoforms may mutually regulate each other's activity and suggested that the non-genomic and genomic estrogen signaling have to be coordinated *in vivo* to maintain normal cell growth. Our finding that SNCG interacts with ER-α66 or ER-α36 depending on the presence of E2 suggested SNCG may function as a coordinator to regulate both genomic and non-genomic estrogen signaling.

We previously developed a specific polyclonal anti-ER-α36 antibody against the 20 amino acids at the C-terminal region that are unique to ER-α36.[14–15] Using this antibody, we found that ER-α36 is expressed in both ER-positive and established ER-negative breast cancer cell lines. Expression of ER-α36 was also demonstrated in clinical breast cancer specimens from both ER-positive and ER-negative breast cancer patients.[42] These data demonstrated a potential biological and clinical relevance of ER-α36 in breast cancer development and progression. Interestingly, we demonstrated that both E2 and its antagonist tamoxifen stimulate ER-α36-mediated ERK1/2 activation (Figure 4D), which was consistent with the previous observations that estrogen antagonists tamoxifen and ICI 182,780 acted as agonists to activate the ERK1/2 signaling pathway through ER-α36.[42] These data indicate that ER-α36 can mediate agonist-like activities of antiestrogens. The antitumor efficacy of tamoxifen has been associated with the inhibition of estrogen binding to the ER-α66 and the subsequent inhibition of receptor transactivation. The mechanism of acquired tamoxifen resistance remains poorly understood. Since tamoxifen treatment up-regulates ER-α36 expression and ER-α36 can mediate agonist-like activities of tamoxifen in stimulation of ERK1/2 pathway, up-regulation of ER-α36 expression and signaling during breast cancer progression may represent an underlying mechanism for tamoxifen resistance. Indeed, we demonstrated that while expression of SNCG renders tamoxifen resistance in the growth of MCF-7 cells; knockdown endogenous SNCG expression in T47D cells makes the cells more responsive to tamoxifen-mediated growth inhibition in colony formation.

This *in vitro* SNCG-mediated tamoxifen resistance is also confirmed *in vivo* in MCF-7 tumor xenograft model. Since SNCG protects ER-α36 expression and stimulates its signaling, we reason that this chaperoning effect on ER-α36 may serve one of the underlying mechanisms for tamoxifen resistance. It is noteworthy to emphasize that SNCG may serve as a tumor-specific chaperone and regulates many signaling pathways, eg, Akt/mTOR signaling (not published), involved in tumor cell survival, and thus SNCG-induced tamoxifen resistance might be mediated by multiple signaling pathways that regulated by SNCG. Nevertheless, our most recent large scale (n = 709) clinical follow-up studies indicate that expression of ER-α36 in ER-α66-positive breast cancer associates with tamoxifen resistance, while ER-α36 expression is not associated with survival in patients with ER-α66 negative tumors.[42]

Hsp90 is one of the most abundant cytoplasmic proteins in unstressed cells, where it performs housekeeping functions and controls the activity, intracellular disposition, and proteolytic turnover of a variety of proteins. So far, at least 100 proteins are known to be regulated by Hsp90, many of which, including HER2, Akt, mTOR, and steroid hormone receptors, are known to be important in the development of cancer.[37–38,43] We previously demonstrated that SNCG participates in Hsp90-based multiple chaperone complex and regulates ER-α66 transactivation.[26–27] One of the critical questions that needs to be addressed is whether SNCG-enhanced and ER-α36-mediated MIES is manifested by Hsp90-based chaperone complex or by its own chaperoning function. Using Hsp90 inhibitor 17-AAG, our data demonstrate that treatment of cells with 17-AAG resulted in a significant down-regulation of E2-induced mTOR activation. However, expression of SNCG completely recovered 17-AAG-mediated loss of mTOR activation. Furthermore, when cells were treated with 17-AAG, while Hsp90 lost it ATPase function and dissociated from its client protein ER-α36, SNCG was still able to physically associate with and chaperone ER-α36. These data suggest that SNCG, which can replace the chaperone function of Hsp90, is an independent chaperone protein and its chaperoning function is not dependent on Hsp90. Thus, SNCG and Hsp90 act cooperatively in ER-α36-mediated MIES.

Unlike the typical chaperone Hsp90, which is essential for a range of indispensable functions in normal tissue, SNCG is not expressed in normal cells but aberrantly expressed in advanced malignant state through epigenetic control by demethylation of CpG sites within SNCG gene,[23] suggesting that SNCG is a more tumor-oriented chaperone. Targeting tumor specific chaperones such as SNCG represents a potential alternative to direct Hsp90 inhibition that may offer greater specificity and an improved side effect profile. Clinical follow-up studies indicate that expression of SNCG in breast cancers renders resistance to endocrine and adjuvant therapy.[24] The present study demonstrated SNCG as a tumor specific chaperone, which can replace the chaperoning function of Hsp90 and regulate membrane estrogen signaling. The study will potentially lead to a new molecular profile of the tumor for the optimal patient selection for endocrine and Hsp90 disruption and a new strategy of combining SNCG targeting with Hsp90 disruption as a novel advantageous approach for treatment of cancer. Since SNCG positively regulates both genomic and non-genomic estrogen signaling, targeting SNCG for treating hormone-dependent breast cancer warrants further investigation.

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